RayBio® Mouse Cytokine Antibody Array G Series 1000

Patent Pending Technology

User Manual (Revised 07062006)

RayBio® Mouse Cytokine Antibody Array G series 1000
(Combination of Arrays III & IV Cat# AAM- CYT-G1000)

RayBio® Mouse Cytokine Antibody Array G Series III (Cat# AAM-CYT-G3)
RayBio® Mouse Cytokine Antibody Array G Series IV (Cat# AAM-CYT-G4)

RayBiotech, Inc.

We Provide You With Excellent
Protein Array Systems And Service

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RayBiotech, Inc.

RayBio® Mouse Cytokine Antibody Array G Series 1000 Protocol

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Cytokine Antibody Arrays are RayBiotech patent-pending technology.

RayBio® is the trademark of RayBiotech, Inc.

I. Introduction

All cell functions, including cell proliferation, cell death and differentiation, as well as maintenance of health status and development of disease, are controlled by many genes and signaling pathways. New techniques such as cDNA microarrays have enabled us to analyze the global gene expression [1-3]. However, almost all cell functions are executed by proteins, which cannot be studied by DNA and RNA alone. Experimental analysis clearly shows a disparity between the relative expression levels of mRNA and their corresponding proteins [4]. Therefore, it is critical to analyze the protein profile. Currently, two-dimensional polyacrylamide SDS page coupled with mass spectrometry is the mainstream approach to analyzing multiple protein expression levels [5,6]. However, the requirement of sophisticated devices and the lack of quantitative measurements greatly limit its broad application. Thus, no simple, cost effective, and rapid method of analysis of multiple protein expression levels has been available to researchers until now.

Our RayBio® Mouse Cytokine Antibody Array is the first commercially available protein array system [7-11]. By using the RayBiotech system, scientists can rapidly and accurately identify the expression profiles of multiple cytokines in several hours inexpensively.

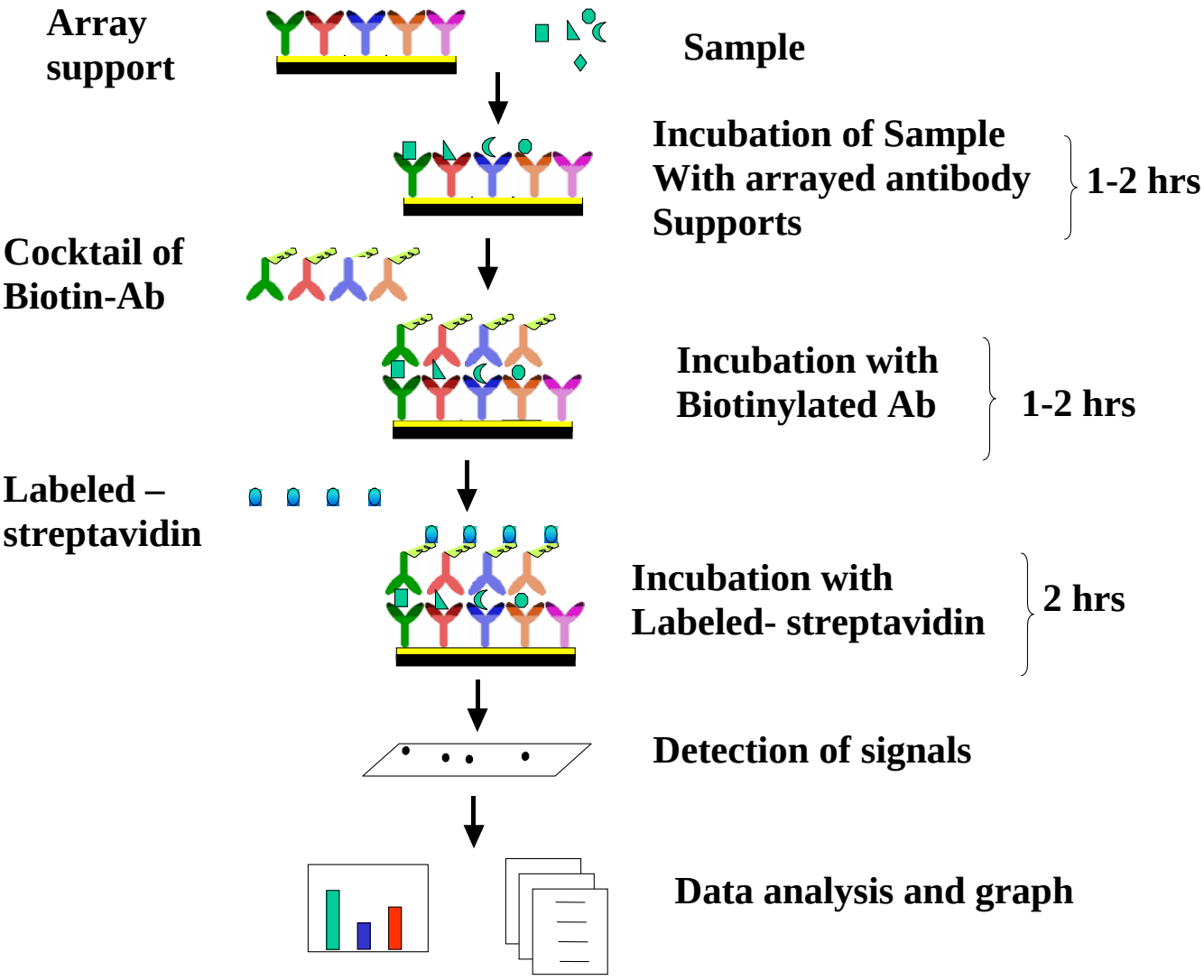
The RayBiotech kit (G series) is a glass chip format. The kit provides a highly sensitive approach to simultaneously detect multiple cytokine expression levels from cell culture supernatant, patient's serum, tissue lysate and other sources. The arrays are manufactured using non-contact arrayer. The experimental procedure is simple and can be performed in any laboratory. The signals from G series arrays are detected using a laser scanner.

The RayBio® Mouse Cytokine Antibody Array G series 1000 can detect 96 mouse cytokines in single experiment. RayBiotech also provides RayBio® Human Cytokine Antibody Array G series 2000 which is the only product available in the market that can detect 174 human cytokines in single experiment.

Pathway-specific array systems allow investigators to focus on the specific problem and are becoming an increasingly powerful tool in cDNA microarray system. RayBiotech's first protein array system, known as RayBio® Mouse Cytokine Antibody Array, is particularly useful compared with the Mouse cytokine cDNA microarray system. Besides the ability to detect protein expression, RayBiotech's system is a more accurate reflection of active cytokine levels because it only detects secreted cytokines, and no amplification step is needed. Furthermore, it is much simpler, faster, environmentally friendlier, and more sensitive.

Simultaneous detection of multiple cytokines undoubtedly provides a powerful tool to study cytokines. Cytokines play an important role in innate immunity, apoptosis, angiogenesis, cell growth and differentiation [12]. Cytokines are involved in most disease processes, including cancer and cardiac diseases. The interaction between cytokines and the cellular immune system is a dynamic process. The interactions of positive and negative stimuli, and positive as well as negative regulatory loops are complex and often involve multiple cytokines.

Here's how it works



II. Materials Provided

Upon receipt, all components of the RayBio® Mouse Cytokine Antibody Array kit should be stored at -20^{0}C. At -20^{0}C the kit will retain complete activity for up to 6 months. Once thawed, the glass chips, Alexa-Flour 555-streptavidin and 2X Blocking Buffer should be kept at –20^{0}C and all other component should be stored at 4^{0}C. Use within three months after reagents have been thawed. Please use within six months of purchase.

- RayBio® Mouse Cytokine Antibody Microarray slides (4/8 subarray III and 4/8 subarray IV)
- Biotin-Conjugated Anti-Cytokines (1/2 tubes, each tube for 4 wells)
- 1,500X Alexa Flour 555-Conjugated Streptavidin (1/2 tubes, each tube for 4 wells)
- 2X Blocking Buffer (4 ml/8ml)
- 20X Wash Buffer I (30ml/60ml)
- 20X Wash Buffer II (20ml/40ml)
- 2X Cell Lysis Buffer (10 ml/ 16 ml)
- Manual

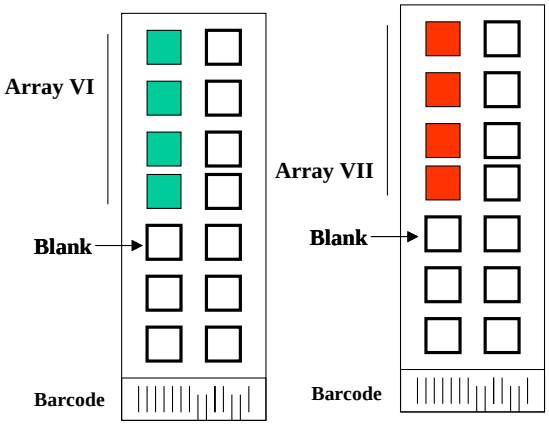
Additional Materials Required

Accessory

RayBio® G series antibody array accessory (including slide incubation chamber, slide frame and adhesive film) (Cat# G-CFF)
Slide incubation chamber (Cat# G-CHA)
Slide frame (Cat# G-FRA)
Adhesive film (Cat # G-FIL)

- Orbital shaker
- Laser scanner for fluorescence detection
- Aluminum foil
- Distilled water
- Plastic box

Layout of Mouse G series 1000



4 arrays in one glass chip

III. Overview and General Considerations

A. Preparation of Samples

- Use serum-free conditioned media if possible.
- If serum-containing conditioned media is required, use serum as control since many types of sera contain cytokines.
- For cell lysates and tissue lysates, we recommend using 1X Cell Lysis Buffer to extract proteins from cell or tissue (e.g. using homogenizer). After extraction, spin the sample and save supernatant for experiment. Determine protein concentration. Dilute 2X Cell Lysis Buffer with H_2O (we recommend adding proteinase inhibitors to Cell Lysis Buffer before use). Prepare relative concentrated lysate since we recommend diluting lysate at least 10 folds with Blocking Buffer for array assay.
- We recommend using
  50 to 100 µl of cell culture supernatant
  or
  50 to 100 µl of original or 5-fold diluted serum or plasma
  or
  20-200 µg of protein for cell lysates and tissue lysates.

If you experience high background, you may further dilute your sample.

B. Handling glass chips

- The microarray slides are sensitive, do not touch the surface. Hold the slides by the edges only.
- Handle all buffers and slides with latex free gloves.

- Avoid breaking glass slide.
- Handle glass chip in clean environment

C. Incubation

- Completely cover array area with sample or buffer during incubation, and cover the incubation chamber with adhesive film or plastic sheet protector to avoid drying.
- Avoid foaming during incubation steps.
- Perform all incubation and wash steps under gentle rotation.
- Cover the incubation chamber with adhesive film during incubation, particularly when incubation is more than 2 hours or 50 μl of sample or reagent is used.
- Avoid cross-contamination from overflowing solution to neighboring wells.
- Several incubation steps such as step 3 (blocking), step 4 (sample incubation), step 8 (biotin-Ab incubation) or step 11 (Alexa Flour 555-streptavidin incubation) may be done at 4^0C for overnight. Please make sure to cover the incubation chamber tightly to prevent evaporation.

IV. Protocol

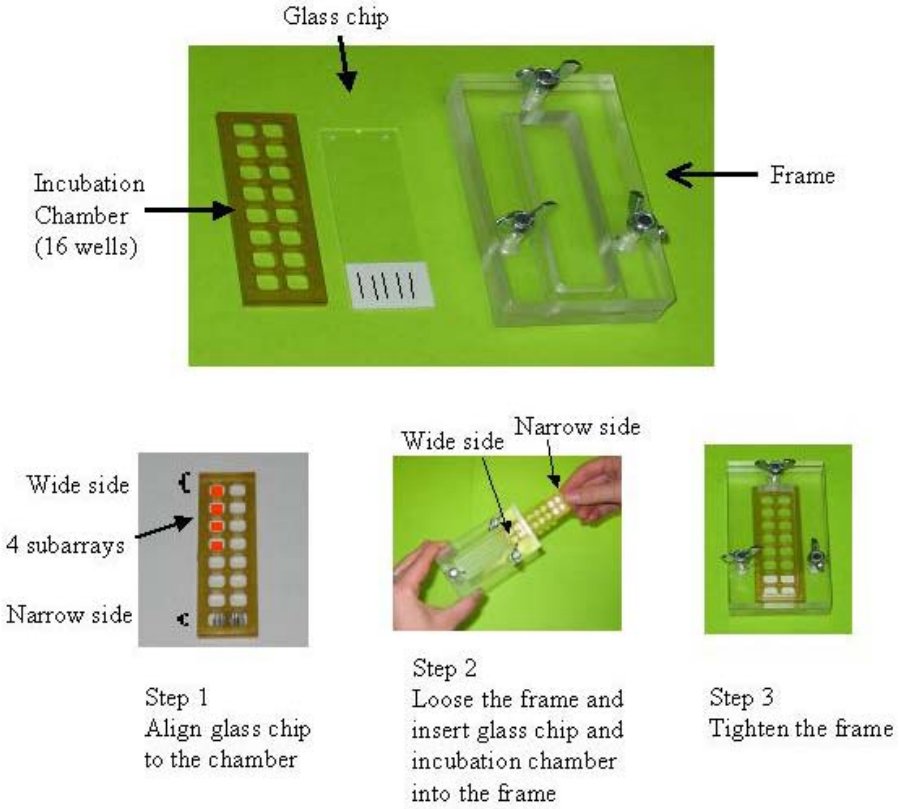
A. Blocking and Incubation

1. Take the glass chip out from the box. Let air dry for 60 minutes.

2. Assemble the glass chip into incubation chamber and incubation frame as shown below.

Step one: align the glass chip (arrays and barcode are at the same side) with incubation chamber (the narrow side of chamber in barcode side) making sure that the array area is faced up and not covered by incubation chamber.
Step two: loose the frame and insert the glass chip and incubation chamber into incubation frame.
Step three: tighten the incubation frame, the glass chip and incubation chamber.

You can practice assembling device using a blank glass slide. After assembled, add 100 µl of dye and let stand for couple hours. If it is not leaking, assemble is successful.



3. Add 100 µl 1 X Blocking Buffer into each well and incubate at room temperature for 30 min to block slides. Dilute 2X Blocking Buffer with H_2O. Make sure no bubbles are in the well.

   Note: only add reagents to wells printed with antibodies.

4. Decant Blocking Buffer from each well. Add 50 to 100 µl of each sample to array III and array IV. Incubate arrays with sample at room temperature for 1 to 2 hours. Dilute sample using 1X Blocking Buffer if necessary.

Note: We recommend using 50 to 100 µl of conditioned media or 50 to 100 µl of original or 2-5 fold diluted serum or plasma or 10-200 ug of protein for cell lysates and tissue lysates. ***Dilute the lysate at least 10 folds with 1 X blocking buffer to make a total volume of 50 to 100 µl. Make sure there is no bubble in the wells.***

Note: The amount of sample used depends on the abundance of cytokines. More sample can be used if signals are too weak. If signals are too strong, the sample can be diluted further.

Note: Incubation may be done at 4^0C for overnight.

Note: when transfer IC, use 0.1 μl to 2 μl pipettor.

5. Decant the samples from each well, and wash 5 times with 150 μl of 1X Wash Buffer I at room temperature with gentle shaking. 2 min per wash. Dilute 20X Wash Buffer I with H_2O. Completely remove wash buffer I in each wash step.

Note: avoid solution flowing into neighboring wells.

6. Wash 2 times with 150 μl of 1X Wash Buffer II at room temperature with shaking. 2 min per wash. Dilute 20X Wash Buffer II with H_2O. Completely remove wash buffer II in each wash step.

7. Prepare working solution for biotin-conjugated antibodies. After brief spinning,

   Add 300 μl of 1x blocking buffer to the Biotin-Conjugated Antibody III tube. Mix gently.

   Add 300 μl of 1x blocking buffer to the Biotin-Conjugated Antibody IV tube. Mix gently.

Note: the diluted biotin-conjugated antibodies can be stored at 4^0C for 2-3 days.

8. Add 70 μl of diluted biotin-conjugated antibodies to each corresponding well (70 μl of diluted biotin-conjugated antibodies III to each subarray III, 70 μl of diluted biotin-conjugated antibodies IV to each subarray IV). Incubate at room temperature for 2 hours.

Note: incubation may be done at 4^0C for overnight.

9. Wash as directed in steps 5 and 6.

10. Add 70 µl of 1,500 fold diluted Alexa Flour 555-conjugated streptavidin (after brief spinning, add 1.5 ml of Blocking Buffer to Alexa Flour 555-conjugated streptavidin tube) to each subarray. Cover the incubation chamber with Adhesive film. Cover the plate with aluminum foil to avoid exposure to light or incubate in dark room.

11. Incubate at room temperature for 1 to 2 hours.

Note: incubation may be done at 4^0C for overnight.

12. Wash with Wash Buffer I **twice** as directed in steps 5.

B. Fluorescence Detection

1. Decant excess Wash Buffer from wells.

2. Disassemble the slide out of the incubation frame and chamber.

3. Place the whole slide in a 50 ml centrifuge tube, add enough Wash Buffer I (about 30 ml) to cover the whole slide and gently shake at room temperature for 10 minutes. Decant Wash Buffer I. Repeat Wash Buffer I once. Wash with Wash Buffer II (about 30 ml) with gentle shake at room temperature for 10 minutes. Or wash using slide chamber. Rinse the slide with distilled H_2O.

4. Remove water droplets by centrifuge at 1,000 rpm for 3 minutes and then let slide dry completely in air at least 20 minutes (protect from light). Make sure the slides are absolutely dry before the scanning procedure.

5. Image the signals using laser scanner such Axon GenePix using cy3 channel.

Note: we recommend scanning slides right after experiment. You also can store the slide at –20^0C in dark for several days. If you do not have a laser

scanner,RayBioteh can provide service for you. Just simply send your slide to us and we will take care of it.

V. Interpretation of Results:

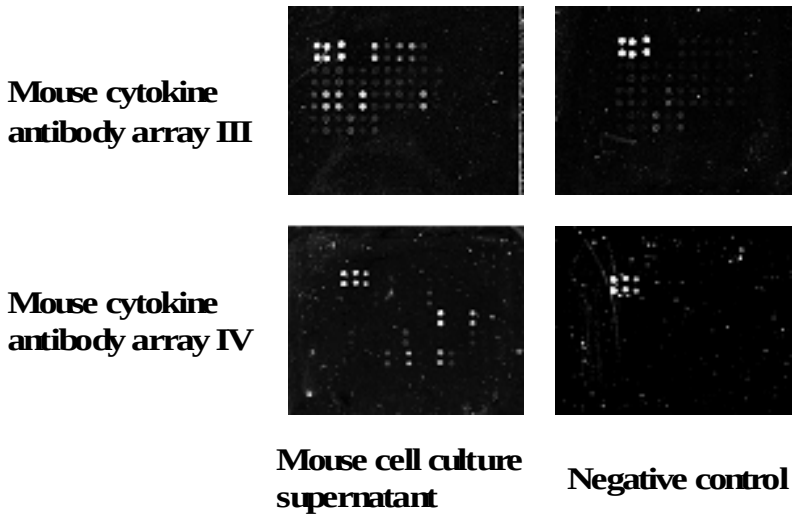
The following figure shows RayBio® Mouse Cytokine Antibody Array G series 1000 probed with different cell culture supernatant. The images were captured using laser scanner. The biotin-conjugated protein produces positive signals, which can be used to identify the orientation and to compare the relative expression levels among the different wells. The internal control (IC) can also be used to normalize the signal intensities among array membranes in different experiments.

The signal intensities obtained from laser scanner can simply be imported into our analysis tool. The analysis tool will help you:

- Locate your signal intensities to antibody array map
- Link the protein to website for more detailed information on the particular protein
- Protein list sorting
- Average signal intensities
- Subtract background
- Normalize the data from different samples
- Obtain protein level comparison charts among different samples

This analysis tool is very simple and affordable, which will not only assist in compiling and organizing your data, but also reduces your calculations to a "copy and paste" step.

Mouse cytokine antibody array G series 1000



If you do not use our **RayBio® Analysis Tool,** you can locate the cytokines by referring to RayBio® Mouse cytokine Antibody Array G series 1000. Please keep in mind that G series 1000 consists of two individual arrays; Mouse cytokine antibody array VI and Mouse cytokine antibody array VII. Refer to corresponding map.

Normalization and comparison

For biomarker discovery or for analysis of large number of arrays, great attention must be paid to the normalization. Our antibody array design includes several controls for normalization and comparison of arrays performing in different membranes and different experiments (for more information please read the reference 17).

Positive control. Positive control is biotinylated protein. It can be used to normalize the streptavidin incubation step. If the positive signals from different array membranes are similar, positive control is a simple and effective way for normalization.

Negative control. Negative control is BSA. Normally, it should only give a background reading.

RayBio® Mouse Cytokine Antibody Array G series 3 (62)

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	POS 1	POS 2	POS 3	NEG	NEG	Axl	BLC	CD30 L	CD30 T	CD40	CRG-2	CTACK	CXCL16	Eotaxin
2	POS 1	POS 2	POS 3	NEG	NEG	Axl	BLC	CD30 L	CD30 T	CD40	CRG-2	CTACK	CXCL16	Eotaxin
3	Eotaxin-2	Fas Ligand	Fractalkine	GCSF	GM-CSF	IFN γ	IGFBP-3	IGFBP-5	IGFBP-6	IL-1α	IL-1 beta	IL-2	IL-3	IL-3 Rβ
4	Eotaxin-2	Fas Ligand	Fractalkine	GCSF	GM-CSF	IFN γ	IGFBP-3	IGFBP-5	IGFBP-6	IL-1α	IL-1 beta	IL-2	IL-3	IL-3 Rβ
5	IL-4	IL-5	IL-6	IL-9	IL-10	IL-12 p40/p70	IL-12 p70	IL-13	IL-17	KC	Leptin R	Leptin	LIX	L-Selectin
6	IL-4	IL-5	IL-6	IL-9	IL-10	IL-12 p40/p70	IL-12 p70	IL-13	IL-17	KC	Leptin R	Leptin	LIX	L-Selectin
7	Lymphotactin	MCP1	MCP-5	M-CSF	MIG	MIP-1α	MIP-1γ	MIP-2	MIP-3 β	MIP-3α	PF-4	P-Selectin	RANTES	SCF
8	Lymphotactin	MCP1	MCP-5	M-CSF	MIG	MIP-1α	MIP-1γ	MIP-2	MIP-3 β	MIP-3α	PF-4	P-Selectin	RANTES	SCF
9	SDF-1α	TARC	TCA-3	TECK	TIMP-1	TNFα	sTNF RI	sTNF RII	TPO	VCAM-1	VEGF	NEG	NEG	NEG
10	SDF-1α	TARC	TCA-3	TECK	TIMP-1	TNFα	sTNF RI	sTNF RII	TPO	VCAM-1	VEGF	NEG	NEG	NEG

RayBio® Mouse Cytokine Antibody Array G series 4 (34)

	A	B	C	D	E	F	G	H	I	J	K
1	POS 1	POS 2	POS 3	NEG	NEG	bFGF	DPPIV/CD26	Dtk	E-Selectin	Fc gamma RIIB	Flt-3 Ligand
2	POS 1	POS 2	POS 3	NEG	NEG	bFGF	DPPIV/CD26	Dtk	E-Selectin	Fc gamma RIIB	Flt-3 Ligand
3	GITR	HGF R	ICAM-1	IGFBP-2	IGF-I	IGF-II	IL-15	IL-17B R	IL-7	I-TAC	Lungkine
4	GITR	HGF R	ICAM-1	IGFBP-2	IGF-I	IGF-II	IL-15	IL-17B R	IL-7	I-TAC	Lungkine
5	MDC	MMP-2	MMP-3	Osteopontin	Osteoporotegerin	Pro-MMP-9	Resistin	Shh-N	Thymus CK-1	TIMP-2	TRANCE
6	MDC	MMP-2	MMP-3	Osteopontin	Osteoporotegerin	Pro-MMP-9	Resistin	Shh-N	Thymus CK-1	TIMP-2	TRANCE
7	TROY	TSLP	VEGF R1	VEGF R2	VEGF R3	VEGF-D	NEG	NEG	NEG	NEG	NEG
8	TROY	TSLP	VEGF R1	VEGF R2	VEGF R3	VEGF-D	NEG	NEG	NEG	NEG	NEG

We also offer Custom Mouse Cytokine Antibody Arrays. You can select the cytokines of interest from the following list and we will produce the customized array at an affordable price. For more information, please visit our website, **www.raybiotech.com**.

Note: IL-12 reacts both IL-12p40 and IL-12p70. IL-12p70 only recognizes IL-12p70.

Abbreviations: IP-10, Interferon-inducible protein-10; LAP, latency associated peptide (TGF-β1); LIF, leukocyte inhibitory factor. MMP, Matrix Metalloproteinase; Pos, positive control; Neg, negative control. All other are used standard abbreviations.

6Ckine	Axl	bFGF	BLC	CD30 L	CD30 T	CD40
CRG-2	CTACK	CXCL16	DPPIV	Dtk	Eotaxin	Eotaxin-2
E-Selectin	Fas	Fas Ligand	Fcg RIIB	Flt-3 Ligand	Fractalkine	GCSF
GITR	GM-CFS	HGF R	ICAM-1	IFN gamma	IGFBP-2	IGFBP-3
IGFBP-5	IGFBP-6	IGF-I	IGF-II	IL-1 alpha	IL-1 beta	IL-2
IL-3	IL-3 Rbeta	IL-4	IL-5	IL-6	IL-7	IL-9
IL-10	IL-11	IL-12 p40	IL-12 p70	IL-13	IL-15	IL-17
IL-17B R	IL-21	I-TAC	KC	Leptin R	LEPTIN(OB)	LIX
L-Selectin	Lungkine	Lymphotactin	MCP-1	MCP-5	M-CSF	MDC
MIG	MIP-1 alpha	MIP-1 gamma	MIP2	MIP-3 alpha	MIP-3 beta	MMP-2
MMP-3	Osteopontin	Osteoporotegerin	PF-4	Pro-MMP-9	P-Selectin	RANTES
Resistin	SCF	SDF-1 alpha	Shh-N	sTNF RII	sTNFRI	TARC
TCA-3	TECK	Thymus CK-1	TIMP-1	TIMP-2	TNF alpha	TPO
TRANCE	TROY	TSLP	VCAM-1	VEGF	VEGF R1	VEGF R2
VEGF R3	VEGF-D					

RayBiotech, Inc., the protein array pioneer company, strives to research and develop new products to meet demands of the biomedical community. RayBio's patent-pending technology allows detection of over 180 cytokines, chemokines and other proteins in a single experiment. Our format is simple, sensitive, reliable and cost effective. Products include: Cytokine Arrays, Chemokine Arrays, ELISA kits, Phosphotyrosine kits, Recombinant Proteins, Antibodies, and custom services.

1. Antibody arrays
   - Cytokine antibody array
     - Human cytokine antibody arrays
     - Mouse cytokine antibody arrays
     - Rat cytokine antibody arrays
   - Pathway- or disease-focused antibody arrays
     - Inflammation antibody array
     - Angiogensis antibody array

Chemokine antibody array
Growth factor antibody array
MMP antibody array
Atherosclerosis antibody array

Antibody analysis tool, software

2. ELISA
3. Cell-based phosphorylation assay
4. Custom antibody arrays
5. Antibody
6. Recombinant protein
7. Cytokine protein arrays

RayBiotech also provides excellent custom service:

1. Antibody arrays
2. Protein arrays
3. Peptide synthesis
4. Production of recombinant protein and antibody
5. Peptide arrays
6. Phosphorylation arrays
7. ELISA

Just simply send your samples and we will do the assay for you.

Technology transfer program
Have you developed technologies or reagents interested to the scientific and research community? RayBiotech can help you commercialize your technologies, reagents and dream.

VI. Troubleshooting guide

Problem	Cause	Recommendation
Weak signal	Inadequate detection	Check laser power and PMT parameters
	Inadequate reagent volumes or improper dilution	Check pipetters and ensure correct preparation
	Short incubation times	Ensure sufficient incubation Time and change sample incubation step to overnight
	Too low protein concentration in sample	Don't make too low dilution Or concentrate sample
	Improper storage of kit	Store kit at suggested temperature
High background	Excess of biotinylated antibodies	Make sure correct amount of antibodies
	Excess of streptavidin	Make sure correct amount of streptavidin
	Inadequate detection	Check laser power And PMT parameters
	dust	Work in clean environment
	Insufficient wash	Increase wash time and use more wash buffer
Uneven signal	Bubbles formed during incubation	Avoid bubble formation during incubation
	Arrays are not completed Covered by reagent	Completely cover arrays with solution

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Note:

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Cytokine protein arrays are RayBiotech patent-pending technology.

This product is intended for research only and is not to be used for clinical diagnosis. Our produces may not be resold, modified for resale, or used to manufacture commercial products without written approval by RayBiotech, Inc.

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